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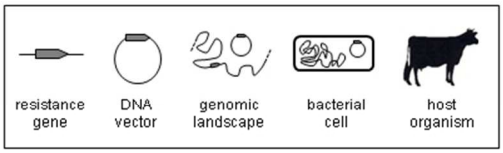
Journal:	*FEMS Microbiology Ecology*
Manuscript ID	FEMSEC-18-09-0521
Manuscript Type:	Perspective
Date Submitted by the Author:	21-Sep-2018
Complete List of Authors:	Smalla, Kornelia; Julius Kühn-Institute, Federal Research Centre for Cultivated Plants, Institute for Epidemiology and Pathogen Diagnostics Cook, Kimberly; USDA_ARS, Micro Djordjevic, Steven; University of Technology, Sydney, Professor, The ithree Institute Klümper, Uli; University of Exeter, Environment and Sustainability Institute Gillings, Michael; Macquarie University, Biological Sciences;
Keywords:	plasmid, insertion element, microbiome, One health, evolution, pathogen



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Perspective – FEMS Microbial Ecology

EDAR 4 Roundtable 1

Environmental dimensions of antibiotic resistance: Assessment of basic science gaps

Kornelia Smalla[1], Kimberley Cook[2], Steven P. Djordjevic[3], Uli Klümper[4], and Michael Gillings[5]

[1]Julius Kühn-Institut Federal Research Centre for Cultivated Plants, Institute for Epidemiology and Pathogen Diagnostics, Braunschweig, Germany, [2]Bacterial Epidemiology and Antimicrobial Resistance Research Unit, U.S. National Poultry Research Center, USDA Agricultural Research Service, Athens, Georgia, USA, [3]ithree institute, University of Technology Sydney, Broadway, Sydney, NSW, Australia, [4]ESI & CEC, Biosciences, University of Exeter, Penryn Campus, Cornwall, UK, [5]Department of Biological Sciences, Macquarie University, Sydney, NSW, Australia.

Correspondence:

Prof Michael Gillings

Biological Sciences

Macquarie University

NSW 2019, Australia

michael.gillings@mq.edu.au

61 2 9850 8199

Keywords: plasmid; insertion element; microbiome; One Health; evolution; pathogen

Abstract

Antibiotic resistance is one of the major problems facing medical practice in the 21st century. Historical approaches to managing antibiotic resistance have often focused on individual patients, specific pathogens, and particular resistance phenotypes. However, it is increasingly recognized that antibiotic resistance is a complex ecological and evolutionary problem. As such, understanding the dynamics of antibiotic resistance requires integration of data on the diverse mobile genetic elements often associated to antibiotic resistance genes, and their dissemination through various mechanisms of horizontal gene transfer between bacterial cells and environments. Most important is understanding of the fate and effects of antibiotics at sub-inhibitory concentration and co-selection. This opinion paper identifies key knowledge gaps in our understanding of resistance phenomena, and outlines research needs that should be addressed to help us manage resistance into the future.

Introduction

Over the past 80 years, antibiotics have revolutionised modern medicine because of their ability to control bacterial infections. However, the use of antibiotics in medicine, and during animal and plant production, imposes strong selection pressures on microbial communities exposed to sub-inhibitory concentration of antibiotics. This has driven the rise of resistance, via mutation and horizontal gene transfer of antibiotic resistance genes (ARGs) in diverse habitats and most prominently in so-called hot spots of horizontal gene transfer (Davies and Davies, 2010; Heuer and Smalla, 2012; Gillings, 2017).

The rise of resistance to all classes of antibiotics poses a major threat to modern health practice. Understanding and controlling the ongoing dissemination of antibiotic resistant bacteria is arguably one of the most pressing tasks for managing human health in the 21st century (Laxminarayan *et al.*, 2013). Like many other pressing human problems, investigating antibiotic resistance involves examining complex, dynamic processes that reflect the global changes in microbial communities induced by humanity (Zhu *et al.*, 2017a).

Mobile genetic elements (MGEs) are an important component of the flexible gene pool of bacterial cells allowing bacterial communities to rapidly adapt to changing environmental conditions e.g. through the presence of antibiotics (Heuer and Smalla, 2012). There is a pressing need to gather comprehensive knowledge about the diversity and dissemination of MGEs that often carry various resistance genes and thus foster their environmental spread. Characterizing complete plasmid sequences and understanding the mechanisms that generate mobile DNAs should be a priority (Chowdhury *et al.*, 2015; San Millan *et al.*, 2016; McKinnon *et al.*, 2018). Understanding the transfer of DNA elements from the environmental resistome into pathogens and commensals is also critical (D'Costa *et al.*, 2006; Gaze *et al.*, 2013; Wellington *et al.*, 2013). By identifying the physical locations and genetic systems where the most rapid change is occurring, we might be able to intervene, and identify potential control points where mitigation strategies might be most effective. However, in many areas, we still lack fundamental information about the origins, dissemination and maintenance of ARGs. To understand these dynamics, we will need to integrate complex data sets that span different biological, temporal and spatial scales and link these with abiotic and biotic environmental factors.

Most importantly, understanding and mitigating the dissemination of antibiotic resistance requires a better understanding of the ecology of MGEs, ARGs and their hosts. Each habitat

is populated by complex and dynamic bacterial communities that differ in their taxonomic composition. These differences in taxonomic composition influence the type and prevalence of MGEs such as plasmids, integrated conjugative elements, phages, transposons and integrons. Land use practices and anthropogenic pollutants in waste streams influence the composition of bacterial communities, potentially selecting lineages that carry adaptive traits and affecting microbial diversity.

In the roundtable discussion at EDAR-4, we identified some key knowledge gaps (Figure 1), and suggested research needs and paths forward to resolving these difficulties.

Gaps in our understanding of baselines and microbial ecology

The modern world is a consequence of historical contingencies (Lowenthal, 2015). We live in an environment where the abundance, distribution and activities of microbial communities, their genes, and the MGEs they carry have been profoundly altered through anthropogenic activity over the last 50 years (Gillings and Paulsen, 2014; Zhu *et al.*, 2017b). This means that we potentially have no baseline of unaffected or pristine samples from some environments with which we can make comparisons.

The dissemination routes of bacteria that carry MGEs and the persistence and mobilization of MGE are not well understood. The relative contributions of transport mechanisms such as of water, wind, dust, migrating wildlife or human activities (tourism, waste management, agriculture) are unclear. In turn, this makes it difficult to estimate rates of increase in abundance and prevalence, especially when some environmental compartments have potentially reached saturated concentrations of antibiotics, metal compounds and ARGs. It has been assumed that the relative abundance of bacteria carrying transferable ARGs is higher in the presence of pollutants (Jechalke *et al.*, 2014), since under these circumstances, plasmid carriage presents a fitness advantage. Certainly, in the absence of such selective pressures, the proportion of the population that carries antibiotic resistance plasmids declines (Jechalke *et al.*, 2013).

In theory, because of the costs of replication and maintenance, plasmids should be lost from their hosts in the long-term due to genome streamlining, regardless of their short-term fitness benefits. Complete plasmid loss occurs when plasmid-free daughter cells outcompete cells that still carry the plasmid. This phenomenon can occur even when under antibiotic selection

pressure if the beneficial resistance gene becomes integrated into the chromosome (Bergstrom *et al.*, 2000; Harrison *et al.*, 2015). However, it was also proposed that plasmid carrying bacterial cells might present only a small proportion of a respective population (multi-cellular behaviour) and that the proportion of these plasmid carrying cells is increased under conditions where plasmid carriage represents a selective advantage (Heuer and Smalla, 2012). Alternatively, compensatory mutations can occur after a plasmid is introduced into a new host, reducing the metabolic burden of plasmid maintenance (Harrison *et al.*, 2016), or turning the cost into a benefit (Loftie-Eaton *et al.*, 2017). These compensatory mutations can increase long-term retention of plasmids. Broad host range plasmids can then spread to diverse new hosts (Klümper *et al.*, 2014, 2017) in which similar co-evolution can occur.

Consequently, it is difficult to estimate the half-lives of resistant bacteria and their cargoes of mobile ARGs because it depends on the particular bacterial species, plasmids, phenotypic traits and environments involved. Knowing the rates of gain and loss in different environmental compartments is essential to assess the effectiveness of mitigation strategies (Figure 1).

There are, however, a few studies that have given us an idea of baselines in the pre-antibiotic era. Examination of resistance genes in archived soil samples (Knapp *et al.*, 2010), samples from permafrost sediments (D'Costa *et al.*, 2011), or in historical plasmid collections (Hughes and Datta, 1983), has given us a glimpse of this pre-antibiotic world. These studies suggest that clinically relevant resistance genes were rare in soils, and were not found on plasmids, prior to the 1940s. We suggest that there should be a concerted effort to examine archived samples, such as soils, seeds, pathology specimens, herbaria and culture collections from before the antibiotic era. This would help us distinguish the naturally occurring resistome (Allen *et al.*, 2010) from those resistome elements whose abundance has markedly increased under human influence (Zhu *et al.*, 2017b, 2017a).

We do know that agricultural practices alter the soil microbiome (Degrune *et al.*, 2017; Hartmann *et al.*, 2017) and thus most likely also the relative abundance of resistance genes and mobile genetic elements in soil. However, the effect of agricultural management on the abundance and diversity of ARGs and MGEs is a clear knowledge gap. In general, those soils that have been exposed to organic fertilizers such as manure, digestates or sewage sludge have readily detectable resistance genes and mobile genetic elements, while these DNAs are below the detection limit in soils without previous history of organic fertilizer application

(Blau *et al.*, 2018b; Wolters *et al.*, 2018). Organic fertilizers are not only a valuable source of nutrients for plant growth but they contain also antibiotics and are a reservoir of ARGs and MGEs (Zhu *et al.*, 2013; Jechalke *et al.*, 2014; Wolters *et al.*, 2016). Furthermore, soil communities that have been subject to manure application display a significantly increased permissiveness towards mobile genetic elements, such as plasmids encoding antimicrobial resistance (Musovic *et al.*, 2014).

Antibiotic residues in manure applied to soil can influence selection and maintenance of resistance genes. This effect will depend strongly on the half-life of the antibiotic, and its bioavailability, which in turn will be dependent on soil type. However, even sub-lethal concentrations of antibiotics are known to select for cells carrying resistance plasmids encoding selective traits (Gullberg *et al.*, 2011, 2014) but might also select for mutations. Multiple mutations in genes that are not associated with resistance can result in highly resistant phenotypes (Wistrand-Yuen *et al.*, 2018). In general, the effects of antibiotics will be further compounded by potential co-selection by contaminating metal compounds, quaternary ammonium compounds and other biocides (Baker-Austin *et al.*, 2006; Jechalke *et al.*, 2014; Song *et al.*, 2017). In this regard, it is worth noting that diverse, multiple drug resistant commensal *E. coli* from pigs can also carry various heavy metal resistance genes (Reid *et al.*, 2017). Understanding the fate of antibiotics in different environmental settings, their interaction with other agents, and their role in selection within environmental compartments is a major gap in our understanding of resistance dynamics (Figure 1).

Bacteria that carry ARGs can occur on the surface of foods that are eaten raw, such as some vegetables. This represents a route of dissemination that deserves more attention, as it represents a direct link between the environment and humans. Transposons and integrons associated with resistance genes have been traced back to leafy vegetables, which have been suggested as the original source of these elements (Ghaly *et al.*, 2017). After incubation of lettuce or cilantro leaves in peptone broth, multiple resistance plasmids were detected in *E. coli* isolates, and in total community DNA obtained from the enrichment (Blau *et al.*, 2018a).

Ingesting resistant bacteria from the environment is directly correlated with a higher risk of gut colonization by these resistant phenotypes. Surfers, for example, are up to 4-fold more likely to be colonised by *E. coli* harbouring $bla_{\text{CTX-M}}$ genes compared to non-surfers, due to an increased ingestion of bathing waters contaminated with resistant bacteria (Leonard *et al.*, 2018). While these resistant bacteria might be lost after 2 to 6 months without exposure

(Kennedy and Collignon, 2010), regular intake of novel resistant bacteria via fresh produce could lead to a constant state of gut colonization. These bacteria can then be transported between continents as residents in the gut of tourists (Bengtsson-Palme *et al.*, 2015).

Based on these studies and others like them, it is clear that general conclusion can often not be drawn as multiple abiotic and biotic factors influence the findings and the ecologies of those systems need to be considered. Physical, chemical, spatial, temporal and biological complexities of natural systems and the ecologies of resistance in those systems preclude a "one size fits all" approach (Durso and Cook, 2014). Scales of environmental studies (i.e., laboratory, experimental/model, and spatial) must also be considered as part of hypothesis driven research. Identification of risk factors, bottlenecks and drivers of resistance may require different strategies for analysis and, ultimately, for remediation. Identifying and communicating applied solutions to critical communities in the field, including farmers, public health, regulatory groups and educators, represents a critical gap that limits implementation of solutions (Figure 1).

Gaps in understanding mechanisms of capture and dissemination

Human attempts to control bacterial growth with antibiotics lead to selection of resistant bacterial lineages, and changes in bacterial community composition. However, a surprising amount of complexity and uncertainty lies behind this apparently obvious statement. What is the relative importance of mechanisms that influence resistance dynamics? Several mechanisms, such as conjugation, require proximity of donor and recipient cell. In other mechanisms, such as transformation, transduction, and DNA transfer by vesicles or gene transfer agents, donors and recipients can be separated temporally and spatially (Gillings, 2017). While conjugation is generally considered to be the most important mechanism, this may simply be because it is more tractable in laboratory and field experiments.

It is abundantly clear that the raw materials for selection of resistance are mutations to existing genes, or horizontal gene transfer events that can confer resistance phenotypes. However, what drives the initial horizontal gene transfer events, what mechanisms are important, where selection occurs, and the units of selection are less clear (Figure 1).

Most likely the importance of different mechanisms depends on the bacterial species, the genes, and the plasmids involved. However, there is also strong evidence that there are

environmental hot spots for resistance gene evolution and horizontal gene transfer (Rizzo *et al.*, 2013; Berendonk *et al.*, 2015). High cell densities, the availability of nutrients allowing bacterial growth and the presence of pollutants are assumed to foster these processes. These conditions can be found within the intestine of humans or animals treated with antibiotics, in sewage treatment plants, animal manures, biofilters and in the rhizosphere. Biofilms are typically also important sites that foster horizontal transfer of ARGs and MGEs (Flemming *et al.*, 2016; Gillings *et al.*, 2017).

To fill the gaps in our limited understanding of the mechanisms of capture and dissemination of ARGs the molecular characterization of MGEs and their ecology is of key importance. Thus sequencing plasmids and complete bacterial genomes shows that plasmids from unpolluted sites carry transposons, IS elements and integrons, but without ARGs. The same plasmid types isolated from hospital environments, sewage sludge, manure or digestates do carry ARGs. Consequently, plasmids appear to be an important means by which bacterial hosts can dynamically and rapidly respond to changing environmental conditions by acquiring advantageous phenotypes. Precisely where the acquisition of resistance genes takes place, and what factors foster these processes remains unclear. While plasmid carriage under conditions of rapid growth might be costly, this might not be the case under environmental conditions. There is a gap in our understanding of horizontal transfer, metabolic costs of plasmids and resistance gene capture under *in situ* conditions and thus research addressing these topics needs to be done under more relevant conditions.

Better understanding the role played by complex interactions between different mobile elements is thus critical. This is perhaps best exemplified by the capture of chromosomally-derived bla_{KLUA} genes from *Kluyvera* species that inhabit the plant rhizosphere, onto conjugative plasmids circulating widely in the Enterobacteriaceae. The bla_{KLUA} genes have close sequence identity to the $bla_{CTX\text{-}M}$ genes that are now globally disseminated in Gram-negative bacterial populations from clinical environments. The insertion element IS*Ecp1* most likely played a key role in mobilising bla_{KLUV} genes onto plasmids (Humeniuk *et al.*, 2002; Zhao and Hu, 2013).

IS*26* is another example for an insertion element that is shaping the accessory gene load of drug resistance plasmids and the structure of class 1 integrons, promoting the formation of clustered resistance gene regions (Cain *et al.*, 2010; Harmer and Hall, 2016; Reid *et al.*, 2017). Copies of IS*26* are known to flank diverse ARGs, creating independently mobile,

compound transposons. The association of IS*26* with class 1 integrons shows how evolutionary events driven by constant selection pressure can assemble a powerful combination of disparate genetic elements with a formidable capacity to capture and express ARGs. Furthermore, IS*26* can influence the formation and ongoing evolution of virulence plasmids via the creation of hybrid plasmids that merge virulence genes with ARGs (Venturini *et al.*, 2010; García *et al.*, 2016; Mangat *et al.*, 2017; Wong *et al.*, 2017). Perhaps of even greater concern is the role played by IS*26*-mediated deletion events in the promotion of plasmid fitness and host range. Newly-acquired plasmids may persist in a host background for long enough to enable IS-mediated modifications to partially alleviate the fitness imposed of carrying such plasmids (Porse *et al.*, 2016). IS*26* is disseminated widely in commensal bacterial populations in the gastrointestinal tracts of food animals (Reid *et al.*, 2017), giving widespread opportunity for such events to occur. These studies are in their infancy and there is a pressing need to understand the role of commensal bacterial populations in the creation and spread of drug resistance elements. Experiments need to be designed to understand these events under *in situ* conditions.

There are still unaddressed issues of scale in the selection of resistant lineages. Selection effects can make themselves felt at scales of the cell membrane, within biofilms, soil particles, local populations, the vertebrate gut, or even at the landscape scale. Each scale has inherently different potentials for generating gradients of selective agents. The targets of selection are also nested at different scales, and include: the resistance gene; the mobile element it may reside upon; the genomic landscape around the resistance gene; the bacterial species involved; and the environmental compartments or the host that carries the bacterium (Figure 1). The intensity and direction of selection may differ across these scales, and it is a priority to tease out these complexities with empirical studies.

Gaps and problems with information and data

It is fair to say that much of the energy devoted to investigating the environmental dimensions of antibiotic resistance has been in descriptive studies. The analysis of total community DNA directly extracted from environmental samples allowed comparative studies on the prevalence and relative abundance of ARGs and MGEs, and at the same time allowed characterization of microbial community composition. These studies have assembled large amounts of data about different sample types, different genes and different species.

Now it is time to pool these data, and this, in turn, will require standardization of nomenclature, metrics and annotation. However, it is also important to note that metagenomic studies, even with deep sequencing efforts, will likely only capture the resistome and mobilome of the most abundant bacteria. Furthermore, it is important to demonstrate functionality and transferability of the resistance genes detected. Therefore, multi-phasic approaches should be used that combine cultivation-dependent and independent methods.

While assembling and analysing these data, we must bear in mind that there is considerable errors and biases in those data that have already been collected. Some of this bias is a historical focus on clinically important species, and on those species amenable to genome sequencing. We need to understand the contribution of yet to be cultured organisms and of the rare microbiome, and this will require metagenomic analyses (Bengtsson-Palme *et al.*, 2017). However, depending on their genetic localization, resistance determinants might be shared by phylogenetically distant taxa. Any one gene can be found in multiple species, in diverse genetic contexts, and linked to transposons and IS elements that can occur in multiple copies in any one genome. This makes accurate assembly of metagenomic data challenging, especially for short read technologies.

Education on microbial ecology, the natural role of antibiotics and how they affect the microbiota

In the last decade numerous discoveries revealed the importance of a diverse microbiota for human well-being and for plant health. Antibiotic treatments disturb these communities and might affect their functionality. Thus prudent use of antibiotics should be an imperative. The rapid spread of ARGs demonstrates that bacteria dynamically respond and adapt to changing environments and stress. The most important message here is that the environmental dimension of antibiotic resistance is closely linked to the veterinary and human dimension of antibiotic resistance. Bacteria carrying ARGs and MGEs are exchanged through multiple paths between the environmental settings and thus the One Health perspective is a logical response to the resistance crisis (Robinson *et al.*, 2016; McEwen and Collignon, 2018).

It is important to note that the causal agents of nosocomial infections were often affiliated to genera such as *Acinetobacter*, *Burkholderia*, *Enterobacter*, *Klebsiella*, *Pseudomonas*, *Stenotrophomonas* or *Serratia* that are typically known as plant associated bacteria (Ryan *et al.*, 2009; García-León *et al.*, 2014). They have the ability to form biofilms and they display

multiple antibiotic resistances, and thus they have enormous selective advantages in the face of human attempts to control bacterial growth. These species potentially serve as important reservoirs of transferable ARGs. The rare plant microbiome also contains *E. coli* carrying transferable multiple resistance plasmids.

For too long, the problem of antibiotic resistance has been thought of as one of individual patients, infected with individual resistant organisms. It is now clear that the resistance crisis, is at its heart, a phenomenon of ecology and evolution. Educating patients, clinicians, farmers and regulators about the environmental dimensions of this problem is essential if we are to make progress. We need to acknowledge that while resistance determinants often originate from environmental compartments, these elements are now so common in human dominated ecosystems, that humans themselves have become a significant source of resistance pollution (Zhu *et al.*, 2017a; Gillings, 2018). Like many of the other environmental crises faced by humanity, antibiotic resistance is now a global problem that requires global solutions.

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Figure legend

Figure 1: Key questions about the dynamics of antibiotic resistance. The ecology and evolution of antibiotic resistance is influenced by a series of nested interactions. Each of these can be viewed from different perspectives, summarized as the elements depicted in the diagram. The key questions that are important for understanding and managing resistance dynamics are summarized on the right hand side. Each of these questions can be applied across the different scales of the illustrated hierarchy.

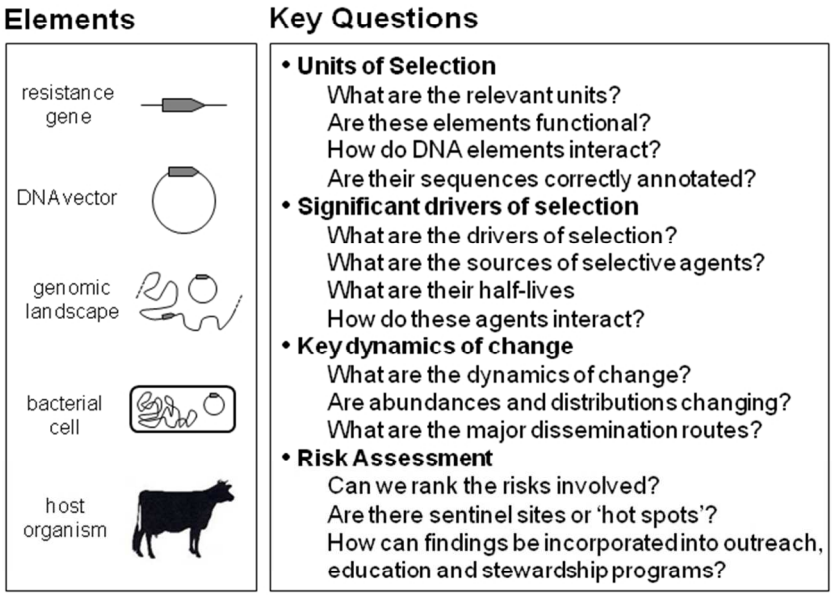

Figure 1: Key Questions about the dynamics of antibiotic resistance

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